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Alternative treatment methods for bovine mastitis: prospects and limitations (review)

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ABSTRACT
Mastitis remains the most common problem of dairy industry despite the preventive measures and treatment schemes being developed. Antibacterial drugs remain first line agents for therapy of the mammary gland inflammatory diseases in animals. Taking into account the risks associated with antibiotic therapy, such as decreased drug effectiveness due to occurrence of bacterial resistant strains, food safety issues, environmental impact and restrictions on the use of antibacterial drugs in veterinary medicine, an increasing number of scientific studies are addressing new therapeutic agents that can serve as an alternative to conventional therapy. The aim of this review is to give an idea of currently available literature data on alternative methods for the prevention and treatment of mastitis in cattle that are not associated with antibiotics. In general, a significant number of *in vitro* studies aimed at finding new effective and safe drugs are yielding promising results. This review describes the following alternative remedies: probiotics, bacteriocins, bacteriophages, phage enzymes (endolysins), nanoparticles, plant extracts, essential oils and immunobiological agents (vaccines). Understanding the mechanisms of their action will allow recommending the best treatment option for mastitis in each specific case. These treatment methods can potentially reduce use of antibiotics and increase animal productivity, however more *in vivo* studies are needed to prove the effectiveness of antibiotics used directly in the conditions of farm settings.

Keywords: review, mastitis, alternative treatments, probiotics, bacteriocins, bacteriophages, endolysins, nanoparticles, herbal extracts, essential oils, immunobiological prevention

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Альтернативные методы лечения мастита крупного рогатого скота: перспективы и ограничения (обзор)

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РЕЗЮМЕ
Мастит продолжает оставаться наиболее распространенной проблемой молочного животноводства, несмотря на разрабатываемые профилактические меры и схемы лечения. Антибактериальные препараты являются основным средством терапии при воспалительных заболеваниях молочной железы у животных. Принимая во внимание связанные с антибиотикотерапией риски, такие как снижение эффективности действия препаратов из-за появления резистентных штаммов бактерий, проблема безопасности пищевых продуктов, воздействие на окружающую среду и введение ограничений на применение антибактериальных препаратов в ветеринарной медицине, все большее количество научных исследований обращается к новым терапевтическим средствам, которые могут стать заменой традиционной терапии. Цель настоящего обзора – дать представление о доступных в настоящее время литературных данных по исследованию альтернативных методов профилактики и лечения мастита крупного рогатого скота, не связанных с антибиотиками. В целом существует огромное количество исследований *in vitro*, направленных на исследование новых эффективных и безопасных средств, которые дают многообещающие результаты. В данном обзоре описаны такие средства, как пробиотики, бактериоцины, бактериофаги, фаговые ферменты (эндолизины), наночастицы, растительные экстракты, эфирные масла и иммунобиологические средства (вакцины). Рассмотрены механизмы их действия, понимание которых позволит рекомендовать наилучший вариант лечения мастита в каждом конкретном случае. Данные методы терапии потенциально могут сократить использование антибиотиков и повысить продуктивность животных, однако требуется больше исследований *in vivo,* чтобы доказать эффективность их применения непосредственно в условиях сельскохозяйственных организаций.

Ключевые слова: обзор, мастит, альтернативные методы лечения, пробиотики, бактериоцины, бактериофаги, эндолизины, наночастицы, экстракты трав, эфирные масла, иммунобиологическая профилактика

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INTRODUCTION

Bovine mastitis, or mammary gland inflammation in livestock, is the most common disease of dairy cows that incurs losses in agriculture. It was established that about 150 different bacterial species/subspecies are capable of causing this disease in cattle. However, more than 95% of mastitis cases are associated with only 10 groups of microorganisms, including both opportunistic and pathogenic ones, depending on their reservoir and transmission method [1]. Such bacteria include *Staphylococcus aureus*, *Mycoplasma* spp., *Streptococcus uberis*, *Streptococcus dysgalactiae*, coliforms and other gram-negative bacteria such as *Escherichia coli* and *Klebsiella pneumoniae*. Other agents, such as *Arcanobacterium pyogenes*, various streptococci (*Streptococcus parauberis*, *Streptococcus agalactiae*, *Streptococcus zooepidemicus*), *Corynebacterium bovis* and *Mycobacterium bovis*, may be involved in the inflammatory process to a lesser extent [2].

Antibiotics are considered the frontline drugs in the treatment of this inflammatory process. However, the issue of antimicrobial residues in animal products and the continuous growth of antimicrobial resistance, together with the possible spill-over of antibiotic-resistant bacteria from animals to humans, results in restrictions on the use of these products in veterinary medicine [3]. The development and introduction of new classes of antibiotics may seem the most obvious strategy, but since 1987 not a single class of antibiotics has been discovered and only derivatives of existing antibacterial drugs have been used [4, 5]. The discovery of several classes of antibiotics in a short period of time has led to their overuse, as well as to a rapid increase in the number of microorganisms with antibiotic resistance genes. In the 1990s such companies as Pfizer, AstraZeneca and GlaxoSmithKline performed studies on potentially new antibacterial targets for antibiotic development, but no suitable candidate was found [6]. Studies of pharmaceutical companies are aimed at modifying existing classes of antibiotics, rather than developing potentially new ones [7]. In this regard, there is currently a need to develop alternative means for the prevention and control of bovine mastitis.

The aim of this review is to give an idea of the latest discoveries related to alternative means, including probiotics, bacteriocins, bacteriophages (phages) and phage enzymes, nanoparticles, herbal extracts, essential oils and immunobiologicals (vaccines) for prevention and treatment of bovine mastitis. Systematized and generalized information and literature sources within the review scope [8–42] are presented in Table 1 in the Additional Files section at: https://doi.org/10.29326/2304-196X-2024-13-3-203-213.

PROBIOTICS

According to modern concepts, mastitis is developed due to imbalance of the mammary gland microbiota, therefore probiotics are viewed as alternative preventive and therapeutic means. Intramammary inoculation of probiotics (lactic acid-producing bacteria) leads to their colonization in the udder [43]. The mechanisms of probiotic activity against pathogenic microorganisms are as follows: adhesion to epithelial cells, aggregation and coagulation, biofilm formation, colonization, production of biosurfactants and/or antagonistic metabolites (organic acids, hydrogen peroxide, bacteriocins), competition for nutrients and/or enzyme production [11]. Probiotic bacteria can be used to control inflammatory processes, especially in the dry season, due to antagonistic activity against mastitis etiological agents and through immunomodulation, namely by influencing the development, differentiation and effector functions of a wide range of subpopulations of immune cells, as well as epithelial cells [11, 44, 45, 46]. In addition to intramammary use, probiotics can also be used as disinfectants, nipple treatments before and after milking [9, 47].

Modern studies are devoted to probiotics used for prevention and treatment of mastitis that contain *Lactococcus lactis*, *Lactobacillus perolens*, *Lactobacillus paracasei*, *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus rhamnosus*, *Schleiferilactobacillus perolens*, *Bifidobacterium breve*, *Bacillus subtilis*.

Many scientists note the potential of probiotics against the most common mastitis pathogens: *S. aureus*, *Staphylococcus epidermidis*, *Staphylococcus chromogenes*, *Staphylococcus intermedius*, *S. agalactiae*, *S. dysgalactiae* and *E. coli* [8], however, these studies were mainly conducted *in vitro*. The mechanism of action of many lactic acid bacteria as probiotics is the inhibition of aggregation of bacterial pathogens to mammary epithelial cells (MAC-T) [19] and the secretion of antimicrobial substances (bacteriocins) [9].

Researchers from Argentina studied 12 species of lactic acid bacteria. Two of them, *L. lactis* subsp. *lactis* CRL 1655 and *L. perolens* CRL 1724, are capable of adhesion to mammary epithelial cells, inhibition and coagulation of 15 *S. aureus*

strains. For mastitis prevention Pellegrino M. et al. recommend intramammary inoculation of these probiotics to cows at dry-off period to activate the immune response by triggering the production of specific antibodies [11].

Another feature of some lactobacilli is the production of their own biofilms. *L. rhamnosus* ATCC 7469 and *L. plantarum* 2/37 strains have the ability to disrupt pathogenic staphylococcal biofilms and replace them with biofilms of their own [12].

The Chinese researchers [15] note the effectiveness of *L. rhamnosus* GR-1-based probiotic for coliform mastitis. This strain of lactic acid bacteria blocks the production of reactive oxygen species and mediates the activation of mitophagy, thereby inhibiting *E. coli*-induced assembly of NLRP3 inflammasome of the family of NLR receptors (NOD-like receptors) that causes apoptosis of mammary epithelial cells. Thus, the use of probiotics promotes the activation of mitophagy and the preservation of mitochondrial cell function.

Qiu M. et al. studied the mechanism of action of *Enterococcus mundtii* H81 in mammary inflammation and for that they used mice models with *S. aureus*-induced mastitis. *E. mundtii* H81 was found to have ability to inhibit *S. aureus* growth. The H81 strain protects the integrity of the mammary epithelial barrier. The results demonstrated that *E. mundtii* H81 reduces pathological damage to mammary tissue by reducing the secretion of proinflammatory cytokines and inhibiting the activation of the signaling pathway of the nuclear transcription factor NF-kB. Consequently, *E. mundtii* H81 may have potential as a promising candidate for the treatment of *S. aureus*-induced mastitis [17].

A number of experiments are aimed at studying the probiotic potential of lactic acid bacteria to better understand how these properties can be used for *in vivo* control of bovine mastitis pathogens.

Intramammary administration of the *L. lactis* probiotic strain proved to be as effective as the conventional antibiotic for the treatment of various forms of mastitis. In this case, the lactococci were completely eliminated from the treated gland after a few days. Many researchers assume that reconvalescence occurs due to induced local inflammation, intensive involvement of leukocytes and stimulation of mammary protection [9, 10, 13].

Catozzi C. et al. [14] investigated intramammary administration of *L. rhamnosus* in buffaloes with a subclinical form of mastitis and observed proinflammatory activity and modification of the milk microbiota. Treatment with *L. rhamnosus* elicited a strong chemotactic response, as determined by a significant increase of leukocytes in milk. Concerning the analysis of the microbiota, the treatment induced the modification in relative abundance of some genera such as *Pseudomonas* spp. and 5-7N15. Initially, there was an increase in the number of somatic cells in milk, but after 6 days the number of somatic cells decreased significantly. A similar response was observed with intramammary infusion of *B. breve* [16]. In this regard, further studies are needed to assess the potential use of GRAS (Generally Recognized as Safe) bacteria as a maintenance treatment against mastitis.

Oral administration of probiotic strains is an alternative for the prevention and treatment of mastitis. As shown by M. Urakawa et al., the introduction of a *B-subtilis* C-3102-based feed additive into the diet leads to a significant reduction in the incidence of mastitis, as well as maintaining the mean value of somatic cells in milk at a level significantly lower than in the control group. Besides, the experimental group had lower levels of cortisol and reactive compounds of thiobarbituric acid, consequently, the cows did not experience oxidative stress. The flow cytometry showed an increase in the proportion of CD4+ T-cells and CD11c + CD172a^{high} dendritic cells in the blood. Dendritic cells are antigen-presenting cells specializing in the absorption and processing of antigen, which play an important role in innate and adaptive immune responses. The data show that *B. subtilis* C-3102 can be used for prevention of bovine mastitis [18].

In general, the studies described above show that probiotic strains have great potential for the development of effective means for the treatment and prevention of mastitis, but their effectiveness in the treatment of the clinical disease form has yet to be determined.

BACTERIOCINS

Bacteriocins are bacterial peptides synthesized on ribosomes that show antimicrobial activity against other bacteria, including antibiotic-resistant strains [44]. Some bacteriocins (e.g., nisin produced by *L. lactis*) are already used for food preservation due to their antimicrobial effectiveness and at the same time a high degree of safety for consumers [3]. In practice, either purified bacteriocins administered directly in their purest form, or viable bacteria producing bacteriocins (mainly lactic acid) are applied [13]. The sensitivity of bacteria to bacteriocins is associated with their interaction with the bacterial cell surface and cell membrane. Cell permeabilization and pore formation represent the main mechanism by which bacteriocins attack target bacteria. Since the surface charge of the plasmolemma and the fluidity of the membrane are two bacterial properties used as targets for bacteriocins, changing these properties makes bacteriocins ineffective, which leads to the development of resistance to bacteriocins [48]. However, this resistance can be overcome by using bacteriocin combinations [49] with each other or with other antimicrobial compounds [50]. In addition, the effectiveness of bacteriocins can be increased through bioengineering. As bacteriocins, unlike antibiotics, are ribosomally synthesized peptides, their amino acid residues can be altered, thus inducing their antimicrobial effect. Bacteriocins are generally divided into 3 classes (Table 2) [48].

A drug containing bacteriocin produced by *Streptococcus equinus* HC5 has been developed for the treatment of bovine mastitis. Bovicin HC5 has some similarities with nisin as regards its mechanism of action, since it is able to bind to lipid II in the cytoplasmic membrane. Brazilian researchers studied the activity of bovicin HC5 against pure or mixed cultures of staphylococci, streptococci and escherichia strains isolated from cows diagnosed with mastitis in various dairy herds, and confirmed its ability to inhibit the growth of more than 80% of the tested streptococci and staphylococci strains, but noted that no antimicrobial effect against *E. coli* strains was observed [20].

Scientists from Thailand studied the antimicrobial potential of the non-ribosomal peptide Pm11, which is produced from pleurocidin, belonging to the family of cationic α-helical peptides found in *Pleuronectes americanus*. In this study, the Pm11 peptide was found to be active against *E. coli* SCM1249, *S. aureus* CM967, *S. agalactiae* SCM1084 and *S. uberis* SCM1310 strains. However, no antimicrobial

Table 2
Classification of bacteriocins

Class		Features	Producers	Example	Mechanism of action
I	Ia	Lantibiotics (< 5 kDa peptides containing lanthionine and β-methyl lanthionine)	*L. lactis*	Nisin	Cell permeabilization and pore formation, lipid II receptor, action against gram-positive bacteria
	Ib	Carbocyclic lanthibiotics containing labyrinthine and labionine	*Actinomadura namibiensis*	Labyrinthopeptin A1	Herpes simplex virus (HSV) and human immunodeficiency virus (HIV)
	Ic	Sactibiotics (sulphur-to-α-carbon-containing antibiotics)	*Bacillus thuringiensis*	Thuricin CD	Gram-positive bacteria
II	IIa	Small heat-stable peptides, synthesized in a form of precursor which is processed after two glycine residues	*Pediococcus pentosaceus*, *Pediococcus acidilactici*, *Lactobacillus sakei*	Pediocin PA-1, sacacins A and R, leukocin A	Cell permeabilization and pore formation, mannose permease receptor. Active against gram-positive and gram-negative bacteria, active against listeria
	IIb	Two-component systems: two different peptides required to form an active poration complex	*L. lactis* subsp. *cremoris*, *L. plantarum*	Lactococcins G, plantaricin EF and plantaricin JK	Cell permeabilization and pore formation, UppP receptor (undecaprenyl pyrophosphate phosphatase), action against gram-positive bacteria
	IIc	Circular bacteriocins	*Lactobacillus gasseri*, *E. faecalis*, *Lactococcus garvieae*	Gassericin A, enterocin AS-48, garvicin ML	Cell permeabilization and pore formation, ABC receptor transporter, action against gram-positive bacteria
	IId	Unmodified, linear, leaderless, nonpediocin-like bacteriocins	*Lactobacillus salivarius*, *L. lactis* subsp. *lactis*	Bactofencin A, LsbB	Cell permeabilization and pore formation, metallopeptidase receptor, action against gram-positive bacteria
III		Large molecules sensitive to heat	*Lactobacillus crispatus*, *Lactobacillus helveticus*, *E. faecalis*	Helveticin M, helveticin J and enterolysin A	Cell permeabilization and pore formation, action against gram-positive and gram-negative bacteria

activity was observed against the *Klebsiella* spp. SCM1282 strain due to the presence of an extracellular polysaccharide capsule in these microorganisms. When the peptide interacts with the bacterial capsule, its structural changes occur, causing sequestration and preventing the peptide from reaching its pathogen membrane target [21].

Garvicin is class II bacteriocin produced by *L. garvieae* strains [24]. Norwegian researchers identified the inhibitory ability of garvicin KS against *Acinetobacter baumannii*. When used in combination with nisin, garvicin also inhibits *S. aureus* growth [25].

In another study, Brazilian scientists studied the antagonistic activity of aureocin 4181, a staphylococcin produced by *S. aureus*. This bacteriocin has proven effective against a wide range of gram-positive bacteria, including other strains of staphylococci and streptococci [26]. The bactericidal mode of action of aureocin is associated with the destruction of cell membranes of mastitis pathogens [51].

Bactofencin A was isolated from gram-positive *L. salivarius* [52] and demonstrated inhibitory activity against *S. aureus* and *Listeria monocytogenes* by acting on the bacterial cell wall [22]. Nisin A, a lantibiotic produced by *L. lactis*, exhibits broad-spectrum activity against gram-positive bacteria. Its mode of action is based on the destruction of the bacterial cell wall by pore formation and inhibition of the biosynthesis of important cell wall precursors. *Lactobacillus reuteri* generates an active aldehyde known as reuterin in the presence of glycerin. This compound was found to be effective against a wide range of gram-positive and gram-negative bacteria because it causes oxidative stress in cells. Several studies were aimed at evaluating the potential of reuterin as a food preservative [53] and disinfectant [54]. Canadian scientists studied the antibacterial effect of bactofencin A, nisin and reuterin bacteriocins both individually and in combination, using them as a means to treat udder nipples before and after milking. The conducted research showed that the use of bactofencin A did not reduce the amount of staphylococci and streptococci on the surface of udder nipples; nisin and reuterin, on the contrary, reduced bacterial contamination. When these bacteriocins were used in combination, the most pronounced antibacterial effect similar to the biocidal action of nisin and iodine was observed. Thus, the combined use of several bacteriocins has many advantages [23]. Xu X. et al. demonstrated that lower concentrations of antimicrobials with synergistic effects are needed to inhibit bacterial growth [55]. Consequently, it reduces treatment costs and risks of adverse effects caused by drug toxic effect [23]. In addition, bacteriocins can be used in combination with antibacterial drugs. For example, nisin A increases the activity of cephazolinum, thereby reducing the dose of the antibiotic in the mastitis treatment. This combination is effective against *S. aureus*, *S. intermedius*, *S. agalactiae*, *S. dysgalactiae*, *Enterococcus faecalis* and *E. coli* [24].

The rapid discovery of new bacteriocins, their development and combination with other bactericidal agents inevitably leads to increased resistance to these drugs. The potential hepatotoxicity of these bacterial peptides should also be taken into account [48]. In general, various approaches are to be considered to solve the issue of resistance and reduce the toxicity of bacteriocins, which have great potential as bioconservants and therapeutic agents.

BACTERIOPHAGES

Bacteriophages (phages) specifically infect bacteria, resulting in either lysis of the bacterial agent (lytic or virulent phages), or in lysogeny – the integration of the bacteriophage's genetic material into the bacterial chromosome

of the host (moderate or symbiotic phages) [56]. Bacteriophages, due to the specificity of their action, cause minimal disruption of the normal microbiome of animals, thereby not causing dysbiosis [57]. Such selectivity of bacterial targets by phages is achieved by recognizing specific receptor proteins located in the bacterial cell wall, on which the phage is adsorbed using specialized fibrils, after which bacteriophages penetrate and release their genetic material in the bacterial cell [58]. As a rule, phages of most *S. aureus* strains interact in the cell wall with teichoic acid, which differs from other acids inherent in coagulase-negative staphylococci [59]. For studies aimed at searching bacteriophages acting against one of the main pathogens of mastitis – *S. aureus*, the following main domains located in endolysin sequences are used: cysteine, histidine-dependent amidohydrolase/peptidase (CHAP), amidase 2 (N-acetylmuramoyl-L-alanine amidase) and SH3b for recognition of the cell wall of the pathogenic agent [60].

Following successful adsorption and penetration into the cell, lytic phages capture the mechanism of bacterial DNA replication to synthesize their own genetic material and structural proteins during the latent period. The duration of the period required to start synthesis varies for bacteriophages acting against bovine mastitis pathogens and can be 5 (*E. faecalis*), 10 (*S. aureus*), 20 (*Pseudomonas aeruginosa*) or 30 minutes (*S. agalactiae*) [61, 62, 63, 64]. Subsequently, after viral synthesis, numerous phage particles are assembled and eventually released by the lysis of the host through a combined activity of the endolysin and holin enzymes that degrade the bacteria cell wall [57]. In case of bovine mastitis, the number of phage particles synthesized and released per bacterial cell varies from 20 to 100 PFU/cell (plaque-forming units per 1 cell) for approximately 175 minutes [61, 62, 63, 64]. The ability of lytic phages to eventually lyse bacteria and replicate after infection ensures the destruction of bacterial pathogens, as well as a constant increase in the concentration of infectious phages (auto-dosing) at the site of infection [65]. In addition, the short replication time demonstrated by phages makes it possible to shorten the drug development timeline, providing the opportunity for rapid individual treatment aimed at specific bacterial strains [57].

Many studies have noted a significant decrease in bacterial load during exposure of phages against pathogenic agents that cause mastitis [27, 28, 29, 30, 31]. However, the resistance was detected within two hours after phage treatment, as evidenced by the resumption of bacterial growth after lysis, which may adversely affect therapeutic efficacy [28]. In order to limit the development of resistance and lysogeny, increase the specificity of the target bacteria and raise lysis efficiency, it is possible to optimize the composition of the phage cocktail [66, 67].

For instance, I. Titze and V. Krömker investigated the efficacy of bacteriophage mixture with *L. plantarum* on *S. aureus* strains isolated from the milk of cows with mammary gland inflammation. The phage cocktail, as well as its combination with lactic acid bacteria, demonstrated high antimicrobial activity against *S. aureus* during a 24-hour incubation period at 37 °C. Statistical calculations showed that only a bacteriophage mixture had a significant effect on the growth rate of *S. aureus* [32].

The Chinese researchers assessed the antibacterial activity of bacteriophage mixtures experimentally. For this purpose, eight lactating Holstein cows were selected and randomly divided into four groups, two animals per each group. Three groups of cows were intramammarly inoculated with 60 CFU *E. coli* ECD2 suspended in 1 mL of pyrogen-free phosphate buffer saline solution (PBS). Phage cocktails containing SYGD1, SYGE1, and SYGMH1 were prepared by mixing the three phages at a 1:1:1 ratio with a primary concentration of about 10^{10} PFU/mL. The mixture was 100-fold diluted with PBS. One group was intramammarly inoculated with 5 mL ceftiofur sodium (600 mg/mL), the second group was intramammarly inoculated with 5 mL phage cocktails (1×10^8 PFU/mL), the third group was intramammarly inoculated with 5 mL PBS only. All products were administered once a day for three consecutive days. The fourth group, as a control group, was neither inoculated nor treated. All three bacteriophages showed promising results as antimicrobial agents, especially when used in a cocktail, such therapy can reduce the number of bacteria, somatic cells and inflammatory factors, alleviate the symptoms of mastitis in cattle and achieve the same effect as with antibiotic treatment [33].

Pathogens causing mastitis are capable of forming biofilms, which limits the access of antibiotics to bacteria [68, 69, 70]. However, phages can prevent biofilm formation or penetrate bacterial pathogens *in vitro* and *in vivo*, which indicates the possibility of their use as an independent treatment or in combination with antibiotics to increase therapeutic effect [28, 69]. In a study by Iranian scientists, bacteriophage M8 showed noticeable lytic activity against all tested types of *S. aureus* (multi-resistant, methicillin-resistant and biofilm-forming strains). This bacteriophage, alone or in combination with other phages and antibiotics, has the potential of being a therapeutic option for intractable inflammatory mammary diseases caused by *S. aureus* [34].

The results of many *in vitro* and *in vivo* studies show that phage therapy is a promising alternative to antibiotics for the treatment of mastitis in cows, and in combination with antimicrobials will reduce the dose of the latter or shorten the period of treatment [71]. However, the efficacy of phage therapy is limited due to their strict specificity against certain combinations of mastitis pathogen strains and the need to use several phages to control a variety of bacterial pathogens. Phage therapy is most effective when the target pathogen is readily available and present in large quantities [72].

PHAGE ENZYMES

One of the ways to handle challenges of phage therapy may be the use of purified products of phage genes, such as lysines. Endolysins (amidase, endopeptidase, glucosidase and transglycosylase), commonly known as enzybiotics, are mureolytic enzymes that are synthesized at the end of the phage lytic life cycle [73]. They impact peptidoglycan bonds and lyse bacteria from the inside, promoting the release of new phages. Endolysins have a wider antibacterial spectrum compared to phages. In addition, they can also lyse bacteria when used exogenously. Endolysins are specific, highly active and carry a lower risk of developing resistance [74].

The well-studied and most active lysines include streptococcal-specific lysine PlyC obtained from bacteriophage C1. Although almost all gram-positive endolysins described to date are encoded by a single gene, the endolysin PlyC of the C1 phage of group A streptococcus

is the only example of a multimeric lysine consisting of two different gene products: PlyCA and PlyCB. One PlyCA subunit with enzymatic activity and eight PlyCB subunits that make up the cell wall binding domain form a complete PlyC complex, which is an endolysin with the highest activity, just one nanogram is enough to destroy 10^7 CFU of various streptococcal species in a few seconds [3, 35, 75].

Schmelcher M. et al. studied the possibility of using endolysins of *λ*SA2 and B30 streptococcal phages as antimastitis agents in 2015. Lysine *λ*SA2 showed high activity in cow's milk against *S. dysgalactiae*, *S. agalactiae* and *S. uberis*, whereas lysine B30 was less effective. Both enzymes significantly reduced the concentration of all three types of streptococci in the mouse mastitis model (with the exception of B30 relative to *S. dysgalactiae*). It is worth noting that the synergistic effect found for the two enzymes *in vitro* was not observed in the mouse model. In general, the results obtained demonstrate the potential of endolysins for the treatment of streptococcus-induce bovine mastitis [36].

A study by the Chinese scientists has shown that LysKΔamidase endolysin is able to inhibit 71 methicillin-sensitive and 66 methicillin-resistant staphylococcus strains isolated from the milk of cows with mastitis. The wide antistaphylococcal *in vitro* activity of this enzyme, including that against multidrug-resistant staphylococci and biofilm-producing staphylococci, indicates that LysKΔamidase can become a means of therapy for intractable inflammatory diseases of mammary glands [37].

However, the number of clinical studies on the use of endolysins for the treatment of bovine mastitis is limited. In one of such experiments J. Fan et al. intramammarly administered 20 mg of endolysin Trx-SA1 to cows at the initial stage of clinical mastitis once a day for 3 days. In 60% of cases, milk samples demonstrated decrease in *S. aureus* total count and the number of somatic cells [38].

Despite the promising prospects for the use of endolysins as a therapeutic means for mastitis, their use requires further study as there are certain limitations. For example, repeated administration of lysing proteins leads to the formation of immunoglobulins against the inoculated phage enzymes, which limit the antimicrobial activity of the latter [44]. In addition, most endolysins are not active against gram-negative bacteria, since the outer membrane protects the underlying carbohydrates and peptidoglycan from direct contact with lysines. Nevertheless, one of the main advantages of using bacteriophages and phage endolysins is their ability to eliminate antibiotic-resistant pathogens against which conventional therapeutic methods are ineffective [38].

NANOPARTICLES, PLANT EXTRACTS AND ESSENTIAL OILS

In addition to the above-mentioned means of mastitis therapy and prevention, relatively new control strategies include the use of nanoparticles, herbal extracts and essential oils.

Nanoparticles have broad-spectrum antimicrobial potential and do not affect the development of resistance in bacteria. The antimicrobial effect of metal nanoparticles is explained by: 1) release of the resulting active oxygen; 2) peroxidation of bacterial proteins and lipids; 3) penetration of carbohydrates into bacterial cells; 4) degradation of microbial DNA; 5) damage to the cell membrane and, as a result, an increase in its permeability [76, 77]. After exposure of nanoparticles on bacteria, a decrease in lactate dehydrogenase activity and adenosine triphosphate levels was observed, which indicates ineffective energy regulation in mastitis pathogens. There is also downregulated gene expression in pathogens, including genes encoding glutathione (GSH), glutathione S-transferase (GST), superoxide dismutase (SOD) and catalase (CAT), which induces bacterial death [77]. The results obtained during the pilot studies showed that copper nanoparticles inhibit *S. aureus* growth and exhibit minimal toxicity to fibroblast cell lines at a concentration of 6.25 μg/mL. Intramuscular administration of copper nanoparticles to rats with staphylococcus-induced mastitis turned out to be more effective than gentamicin injections; these conclusions were made based on clinical signs, results of total bacterial load and the study of histological specimens [39].

However, as the use of nanoparticles in mastitis therapy has not yet become widespread as an alternative to the classical approach using antibiotics, many researchers prefer combination therapy including nanoparticles and antimicrobials. It is already known that intramammary administration of the drug with nanosilver and ceftiofur has therapeutic efficacy up to 93.33% of cases. This combination can also be used for preventive purposes, for example before calving [78].

The use of plant extracts and essential oils in the treatment of mastitis is a fairly promising area of research, since, compared with antibiotics, these drugs have a natural composition, they do not have severe side effects [79], and plant components do not participate in bacterial resistance in bacteria after prolonged exposure [80]. This method of mastitis treatment in food-producing animals has been known for a long time, extracts of plants such as *Taraxacum mongolicum*, *Lonicera japonica*, *Viola patrinii*, *Folium isatidis*, *Angelica dahurica*, *Coptis chinensis*, *Phellodendron amurense*, *Rheum officinale*, *Scutellaria baicalensis* have been used in traditional Chinese medicine, showing detoxifying, anti-inflammatory and antibacterial effects [3]. However, the mechanism of action of most extracts and essential oils has not been fully clarified [81]. For example, the antimicrobial activity of such drugs is provided by various plant secondary metabolites, among them are: geranyl acetate, eugenyl acetate, trans-Cinnamaldehyde, menthol, carvacrol, thymol, geraniol, eugenol, p-cimene, limonene, terpinene and carvone [82].

The mechanism of action of plant extracts and essential oils on a bacterial cell is probably associated with degradation of the cell wall, damage to the cytoplasmic membrane and its proteins, release of cellular contents, coagulation of the cytoplasm and destabilization of proton driving force [82]. Gram-positive bacteria are more susceptible to essential oils as compared to gram-negative ones, possibly because the latter have a thick layer of lipopolysaccharides in the outer membrane that covers the cell wall, limiting the diffusion of hydrophobic compounds [83].

In fact, many studies have confirmed the effectiveness of these plant derivatives against bacteria that cause inflammation of bovine mammary gland. For example, the scientists from Pakistan studied the antibacterial effect of *Allium sativum*, *Bunium persicum*, *Oryza sativa* and *Triticum aestivum* against strains of the most common pathogens of mastitis, such as *S. aureus*, *E. coli* and *K. pneumoniae*. It was found that all extracts significantly inhibit ($p < 0.01$;

$p < 0.05$) bacterial strain growth [40]. In another study, M. F. Cerioli et al. observed the inhibitory effect of *Minthostachys verticillata* essential oil and limonene on biofilm formation in *E. coli*, *Bacillus pumilus* and *Enterococcus faecium* strains isolated from cattle with signs of mammary gland inflammation. The results showed that the effect of essential oils is more apparent than limonene, which did not show bactericidal activity against *E. faecium* [41]. The Serbian scientists studied the antibacterial activity of *Thymus vulgaris* L., *Thymus serpyllum* L., *Origanum vulgare* L. and *Satureja montana* L. essential oils in the treatment of mastitis. For that, lactating cows of the experimental group received 15 mL of a drug containing essential oils into the mastitis-affected udder lobes. When comparing the total bacterial load in milk samples before and after treatment, it turned out that this drug effectively inhibited growth of *Staphylococcus* spp., *Streptococcus* spp., *Klebsiella* spp., *Proteus mirabilis*, *E. coli*, *S. uberis*, *Serratia marcenses*. The dominant compounds in the resulting product were thymol and carvacrol. The quantification of these two compounds in the evaluated biological samples showed that their withdrawal period is 24 hours [42].

However, there are some aspects that are considered as limiting the use of plant extracts and essential oils for the treatment of bovine mastitis. Thus, research should be aimed at finding industrial extraction methods, methods for converting plant extracts or essential oils into concentrated and homogeneous products and ways to use such drugs.

VACCINE PREVENTION

In many countries, the disease freedom of agricultural organizations is ensured by means of autogenic vaccines used mainly for the prevention of diseases caused by *S. aureus* and *Mycoplasma bovis*, and, to a lesser extent, by *S. uberis*. These vaccines are prepared based on isolates recovered on site from cows with mastitis, and then administered to the entire herd. In addition, commercial autogenic vaccines against mastitis are also available, for example, Bestvac® based on *S. aureus* strains (IDT, Germany) [84]. Mono- and polyvalent vaccines are also commercially manufactured. The vaccines against coliform mastitis available on the market include: 1) Enviracor™ J-5 contains the mutant strain J-5 *E. coli* (Zoetis, USA) and is administered subcutaneously three times (at drying off, in 4 weeks after drying off and within 2 weeks after calving); 2) J-VAC® *E. coli* contains bacterin-toxoid *E. coli* mutant strain J-5 (Merial, Germany) and is administered subcutaneously or intramuscularly twice (at drying off and in 2–4 weeks); 3) ENDOVAC-Dairy® is a bacterin toxoid derived from the mutant Re-17 *Salmonella typhimurium* (Endovac Animal Health LLC, USA), it provides protection against pathogens such as *E. coli*, *Salmonella*, *Pasteurella* and *Mannheimia*, is administered intramuscularly twice (at drying off and in 2–3 weeks). Vaccines effective against *S. aureus* are also available, for example Lysigin® (Boehringer Ingelheim, Germany), which is injected subcutaneously into the intramammary lymph node three times (4 weeks and 2 weeks prior to calving, with revaccination in 6 months).

Besides autogenic vaccines, inactivated ones are also used for mastitis prevention. The STARTVAC® multivalent vaccine (Hipra, Spain) contains *E. coli* (strain J-5) and *S. aureus* CP8 (strain SP 140) [85] and is administered intramuscularly thrice (45 days before calving, 10 days before calving, 62 days after the second vaccination). As for domestic products, there is MastitVak-EVA vaccine (ARRIAH, Vladimir) consisting of inactivated bacterial cells of *S. agalactiae*, *S. dysgalactiae*, *S. uberis*, two strains of *S. aureus*, *Staphylococcus hyicus* and two *E. coli* strains. For developing a primary immune background against the main clinically significant mastitis pathogens, it is recommended to vaccinate heifers starting from 20–22 weeks of age, and revaccinate after 2 weeks, followed by revaccination every 6 months.

Despite the fact that various commercial vaccines against mastitis are available, none of them provide complete protection, and moreover, are cost-effective [43]. There is evidence that the studies conducted in this respect did not reveal significant differences in mastitis occurrence and the somatic cell count in the milk of the control group cows and experimental groups of vaccinated animals [86]. The insufficient protective potential can be explained by many factors: age, health status, and different immune responses in individual animals depending on genetic and environmental conditions [3, 87, 88].

CONCLUSION

To sum it up, most of the literature data presented has shown the possibility of using new therapeutic approaches to overcome the limitations of traditional antibiotic-based therapy. However, for most of the alternative testing methods, only *in vitro* tests were conducted; additional, mainly *in vivo* tests, are not available yet, though they are critically important and necessary. The considered treatment methods probably will not be able to completely replace antibiotic therapy. The most rational solution would be to combine conventional antibiotic treatment schemes with new alternative approaches, this will reduce the duration of antibiotic use and the withdrawal period for milk, which, in turn, will increase productivity and reduce the likelihood of resistant bacterial strains. It should be considered that the prevention of bovine mastitis is achieved through improving the quality of life and conditions of animals, disinfection of udder nipples before and after milking, timely maintenance of milking machines, which are generally accepted measures to prevent the occurrence of new cases of mastitis.

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